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Diverse immunostaining patterns of Mineralocorticoid Receptor monoclonal antibodies

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Abstract

The mineralocorticoid receptor (MR) is a widely distributed ligand activated nuclear transcription factor that is bound by various chaperone proteins that alter its conformation depending upon its location in the cell and whether it is ligand-bound. We describe the development and characterization of new monoclonal antibodies produced against a rat recombinant protein corresponding to aminoacids 5-550 of the MR to produce antibodies that recognize the receptor in specific conformations. Most of the resulting monoclonal antibodies studied were similar to those we produced by immunization with peptide isotopes, however two detected a single band at the appropriate molecular mass as the MR and had distinct immunostaining characteristics in neurons. One labeled cytosolic MR, the other labeled membranes and cytosol, including axons. These antibodies will permit study of the subcellular localization of the MR under various physiological and pathological conditions. We have also confirmed that the MR is highly unstable and requires special handling.

Keywords

Mineralocorticoid receptor; Aldosterone; Monoclonal antibodies

Introduction

1.1. The Mineralocorticoid receptor (MR) is a member of the ligand-regulated superfamily of steroid receptor transcription factors [1-3]. These receptors have several common structural functional domains which include a ligand binding, DNA binding, hinge, and N-terminal region (A/B) [2,4]. Coactivators, corepressors and chaperonins bind to the binding domain and the N-terminal domain to modulate binding to DNA and affect gene expression [2,5,6]. Structurally, the most closely related receptor to the MR is the glucocorticoid receptor (GR) which is 94% homologous in the DNA binding domain and 57% in the ligand

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binding domain, but only 15% in the N-terminal region [4,7]. The MR and other steroid receptors mediate gene expression by binding to hormone response elements as homo or hetero dimers in a ligand-dependent manner [4,8]. The MR has a similar affinity for the mineralocorticoid aldosterone and the glucocorticoids corticosterone and cortisol [1,9]. Although rats and mice synthesize only corticosterone, cortisol is the predominant glucocorticoid in humans and many other mammals, including other rodents. Specificity for the MR is exerted in some transporting epithelia by the co-expression of the 11 β -hydroxysteroid dehydrogenase 2 enzyme (11-HSD2) which inactivates cortisol or corticosterone [10,11]. Other mechanisms by which the MR may attain some degree of specificity for aldosterone includes a faster dissociation rate of cortisol from the receptor compared to aldosterone [12] and cell-specific expression of co-activator proteins that preferentially bind the MR:aldosterone complex [13].

1.2. MR is expressed in multiple organs and modulates various functions distinct from electrolyte and water transport which occurs primarily in the kidney, colon, salivary glands and sweat glands. The highest concentration of the MR is in the central nervous system, primarily in the hippocampus where they modulate multiple cell processes and are involved in learning and memory [14]. Aldosterone acts through brain receptors in the hypothalamus, brainstem and amygdala to increase blood pressure and sodium retention [14,15].

1.3. While other members of the steroid receptor family are found primarily in the nucleus, the classical view has been that in the absence of ligand MR and GR are primarily located in the cytoplasm and move into the nucleus within a short period of time after binding to an agonist [16]. Recent studies have shown that the MR resides primarily in the nucleus of the mouse cardiomyocyte even in the absence of ligand [17]. The subcellular localization of the MR has not been addressed specifically, however, in cells that express the 11-HSD2, the MR appears to be tethered to the enzyme in the absence of aldosterone [18]. Non-genomic effects of aldosterone have also been clearly demonstrated [19,20]. While it has been postulated that MR localized in the plasma membrane might mediate these effects [21,22], there is recent compelling evidence that GPR30 might also mediate the non-genomic effects of aldosterone [23].

1.4. We developed antibodies against the MR using several peptides corresponding to portions of the N-terminal domain of the MR which are currently extensively used [24,25]. We are now reporting the development and characterization of monoclonal antibodies against a recombinant protein corresponding to amino acids 5-550 of the MR. While most of these exhibit similar characteristics in western blots and immunohistochemistry as the anti-peptide MR antibodies, some have unique immunohistochemical characteristics that have allowed us to address the subcellular localization of the MR.

Materials and Methods

2.1. Monoclonal antibody development

Monoclonal antibodies were developed against a recombinant N-term portion of the rat MR (amino acids 5-550) generated in *E. coli* as previously described for producing monoclonal antibodies against smaller peptides of the MR [24]. Female Swiss-Webster mice (n=8) were immunized subcutaneously with 25 μ g of recombinant protein in complete Freund's Adjuvant and boosted twice at 3 week intervals with 25 μ g of protein in incomplete Freund's Adjuvant. Two weeks after the last injection, the recombinant protein in aqueous solution was injected intraperitoneally and 3 days later blood and spleen were collected. The spleen cells were divided into two aliquots and frozen in cryoprotectant media (5% DMSO in Iscove with 25% fetal calf serum). Splenocytes from two mice with the highest serum titers as measured by ELISA against the antigen were used to produce hybridomas. One aliquot

representing half a mouse spleen was fused with the mouse myeloma SP2-mIL6 using PEG 1,500 (Roche Diagnostic, Indianapolis, IN) as described [24]. It was then reconstituted in 2 ml of media, mixed thoroughly with 100 ml of HAT media with 5% of high viscosity methylcellulose (Methocel MC, Sigma-Aldrich Chemical Company, St. Louis, MO) containing 25% FetalClone I (Hyclone, Provo, UT), hIL-6 (10 ng/ml) (PeproTech Inc, Rocky Hill, NJ) and Endothelial Cell Growth Supplement (50 µg/ml) (EMD Millipore), and 1.2 ml was plated into 8 x 6-well culture plates [26]. After 10-21 days, individual colonies were handpicked and placed in 96-well plates to grow. Supernatant from these clones was tested by ELISA using 100 ng of recombinant protein immobilized into 96-well high binding plates (Greiner) and detected using a goat anti-mouse-HRP antibody (Jackson ImmunoResearch, West Grove, PA) and ABTS reagent [24]. Positive antibodies were then tested by western blot and immunohistochemistry. Only clones that gave a single band on western blot using rat hippocampus cytosol were evaluated. Clones were subcloned using the methylcellulose media as above.

2.2. Western blots

Whole cell homogenates from M1-rMR cells (M1 cells infected with a lentivirus pFUGW-rMR that expressed the MR constitutively) and cytosol from rat hippocampus were used to test the antibodies in the western blot (wb). The wb was performed using 7.5% polyacrylamide gels, then transferred to PVDF membranes. The membranes were blocked with 1% dry milk in Tris 0.05 pH 7.4 with 0.05% tween-20. Multiple commercially available peroxidase-labeled second antibodies were screened, as the sensitivity for detection was extremely variable. A goat anti-mouse IgG heavy and light chain peroxidase-labeled second antibody (Jackson ImmunoResearch, West Grove, PA) was chosen. The membranes were developed using Pierce WestPico SuperSignal (Thermo Fisher Scientific) and recorded using auto-radiographic film (Fuji film, Thermo-Fisher Scientific).

2.3. Immunohistochemistry

Collection of adrenalectomized and intact rat samples has been described [24]. More recent tissue samples were fixed in buffered formaldehyde, as the previous STF fixative was no longer commercially available. Formaldehyde fixed samples were subjected to antigen retrieval using citrate 0.01 M, pH 6.0 for 20 min in a pressure cooker. Results from tissues from intact rats are shown except where specified.

2.4. Stability of the MR

Many published western blots using various academic or commercial anti-MR antibodies show only a small portion of the blot comprising the band corresponding to the mass of the MR. Our initial experience with MR western blots using heart or hippocampus cytosol were inconsistent, with some showing a single band of the appropriate ~107 kDa mass, others having multiple additional bands that appear to be degradation products. To determine a method to ensure consistency, rat hearts were collected and either immediately frozen in liquid nitrogen and stored overnight at -70C before thawing in ice and processing by differential centrifugation, or immediately cut in pieces, homogenized in ice-cold homogenization buffer with protease and phosphatase inhibitors (Thermo Fisher Scientific) and subcellular organelles prepared by differential centrifugation. An aliquot was separated for measurement of protein and the rest was immediately frozen or diluted with Laemmli buffer (2x) and boiled for 10 min and then frozen at -70C. Western blots were performed on both samples using antibody rMR 64-82 2B7 [24].

All animal use was approved by the G.V. (Sonny) Montgomery VA Medical Center Institutional Animal Care and Use Committee. The Animal facilities are AAALAC accredited.

Results

3.1 Stability of the MR

The MR is very unstable, as shown in Fig 1. Hearts collected and immediately homogenized in ice-cold homogenization buffer with protease and phosphatase inhibitors, subcellular fractions prepared, mixed in Laemmli buffer, and boiled showed a single band on western blot, 1A (antibody rMR 64-82 2B7 [24]). Similar results were obtained if the subcellular fractions were immediately frozen and western blots done later. However, hearts collected, snap frozen in liquid nitrogen, stored overnight at -70°C, thawed in ice, and processed exactly as the others, gave several bands of less than 107 kDa that apparently represent degradation products, 1B. The new MR antibodies gave exactly the same bands as antibody rMR 64-82 2B7, the most sensitive antibody for western blots (not shown). Cells are less problematic than tissues, but still require careful preparation. All subsequent studies were done with freshly processed samples.

3.2 Isolated monoclonal clones

Media from 85 clones, approximately 5% of individual hybridoma clones, detected the MR by ELISA; 66 of these detected it by western blot. We selected 10 clones that gave the strongest signal and a single band at the appropriate mass using cytosol from rat hippocampus as reference.

Fig 2 shows western blot of subcellular fractions of the hippocampus with 6 different representative antibodies. Lane 1 is cytosol, lane 2 is the nuclear fraction. The gels were loaded with the same amount of protein (5 µg), however the film had to be exposed for a longer period of time for antibodies 4G5 and 3C9 to detect the bands, indicating that these antibody were specific, but less sensitive for western blots (Fig 2).

Antibodies 4E4, 5B1, 12A7 and 15D11 produced immunohistochemistry staining pattern similar to those previously described (not shown) [24]. Antibodies 4G5 and 3C9, though not the most sensitive for western blots produced distinct immunohistochemistry staining (Fig 3) as discussed in section 3.4.

3.3 M1-rMR cells that were serum starved overnight and then incubated with aldosterone, spironolactone or aldosterone + spironolactone, subjected to subcellular fractionation, then western blot, confirmed, as previously described [16], that in the absence of ligand most MR is in the cytosol, but it is also clearly detectable in nuclei, albeit at a lower levels (Fig 4). Aldosterone produced a marked increase in MR in the nucleus.

3.4 Immunohistochemistry

Immunohistochemistry by most of the antibodies against the MR recombinant protein detected MR in the cytosol and nucleus, a pattern of staining similar to that described for our anti-peptide antibodies [24] and others [25,27,28]. Antibodies MRN-4E4 and MR-12A7 in Fig 3 show this common pattern of immunoreactivity. Elimination of circulating aldosterone by adrenalectomy did not produce exclusive cytoplasmatic localization of the MR. Immunoreactivity in the absence of ligand was both cytoplasmatic and nuclear and of greater intensity in both sites in adrenalectomized compared to intact rat brains suggesting an increase in MR protein. Antibody rMR-12A7 was used for the comparison between MR immunoreactivity in the cytoplasm and nucleus in adrenalectomized and intact rat hippocampus in Fig 3, however similar results were obtained with other antibodies tested, including the previously described anti-peptide antibodies [24]. Antibody 365-4D6, used for immunofluorescence in the cerebellum of an intact rat in Fig 3, also detected MR in both the cytosol and nucleus, but preferentially stained those in the nucleus. Two antibodies from the

present fusion from animals immunized with the recombinant protein produced unmistakably different immunostaining patterns. Immunostaining of the CNS with antibody 3C9 only stained the cytosol in tissues of both intact and adrenalectomized rats (data not shown). Fig 3 shows 3C9 immunofluorescent staining of MR in the cytosol and plasma membrane of Purkinje cells of the cerebellum, and cardiomyocytes and coronary vessels (Fig 3). Antibody 4G5 stained also the cytoplasm and plasma membranes, including those of neuronal cell bodies and axons (Fig 3).

Discussion

4.1. MR function was initially studied in the kidney and other transport epithelia where it regulates the vectorial transfer of sodium, potassium and hydrogen ions, however it is widely distributed in non-epithelial cells as well, including in the brain where it participates in learning and memory, blood pressure and sodium appetite through receptors located in the hippocampus, hypothalamus, brainstem and amygdala [29-31]. MR in the heart and vessels participate in the development of cardiac fibrosis and cardiovascular damage [32]. the identity of the activating ligand for most MR in non-epithelial tissues remains controversial and mechanisms of actions require further clarification as some effects appear mediated by aldosterone and others by corticosterone or cortisol [32,33].

Fig 1 demonstrates that the MR is unstable during isolation and requires prompt handling and inactivation of enzymes of degradation. This probably explains why most publications only show a narrow section of the western blot. The significant degradation that can occur while obtaining a sample makes it difficult to quantify the receptor by western blot, however with rapid and appropriate technique single bands can be obtained. Processing the sample immediately after harvest in a buffer containing protease and phosphatase inhibitors preserves the MR intact and the sample can then be frozen for future western blots, Fig 1A. Freezing the sample immediately upon harvest before protein isolation results in significant degradation when processed later, even if the frozen sample is kept very cold and protease and phosphatase inhibitors are added during thawing Fig 1B.

4.2 Antibodies 3C9, 4G5 detect MR in cytosol and membranes, and both antibodies, most prominently antibody 4G5, clearly stained axons, very likely their plasma membranes. One of our previously published antibodies, rMR 1-18 6G1, [24] has been used with electron microscopy to detect MR immunoreactivity in nuclear and extranuclear sites including dendrites, dendritic spines and presynaptic terminals of glutamatergic and GABAergic neurons [34]. MR were also observed at postsynaptic membrane densities of excitatory synapses [34]. It is very difficult to demonstrate membrane localization using antibody rMR 1-18 6G1 for regular immunohistochemistry because the pattern outside neurons appear more like non-specific binding. Antibody 4G5 produces very clear staining of axons and membrane and should be more valuable for this purpose (Fig 3). Rapid responses to corticosterone of neurons in hippocampal slices have been demonstrated to be mediated through a membrane-associated MR and do not occur in hippocampal slices prepared from MR knockout mice [35]. The generally accepted paradigm that the ligand-free MR resides in the cytosol until binding its agonist ligand, at which time it is translocated to the nucleus, assumes association with chaperonins and molecular motors within microtubules including HSP90, HSP70, immunophilins and dynein heterocomplexes [36]. Incubation of CV-1 cells expressing a GFP-tagged MR construct with aldosterone demonstrates rapid translocation of MR to the nucleus where they form clusters with chromatin; spironolactone produces a slow translocation to the nucleus and does not form clusters [16]. The differences between this and the current study are that, while the former visualized single cells by fluorescent microscopy, we studied sub-cellular fractions of M1 cells stably expressing the MR by western blot, which detects an average signal from many cells and may be more sensitive.

Recent studies using atomic force microscopy have shown aldosterone binding to endothelial cell membranes [21]. Evidence for a plasma membrane associated MR and its function has been confusing and sometimes contradictory [22,37, 38]. One source of confusion may be that aldosterone also binds to the transmembrane G protein receptor GPR30 at physiological concentrations and corticosterone does not appear to activate the receptor [23].

In conclusion

We have described the development of a new panel of monoclonal antibodies against a recombinant protein corresponding to amino acids 5-550 of the MR with unique abilities to detect the MR in different conformations, thus locations within the cell that should become tools to resolve questions about mechanisms of MR ligand specificity and action. We also demonstrated that the MR is very unstable and requires especial processing before doing a western blot. Attention to the manner in which samples are processed should resolve discrepancies between results reported in the literature.

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Highlights

- Monoclonal antibodies were raised against a recombinant mineralocorticoid receptor.
- A subset of antibodies labeled only cytosol or plasma membranes.
- The mineralocorticoid receptor is highly unstable.

Figure 1.

The left panel shows a western blot of 5 μ g of cytosol from 3 heart samples (lanes 1-3) that were processed immediately after harvest and frozen before western blot analysis. The panel on the right shows a representative western blot from a heart that was snap frozen in liquid nitrogen upon harvest and stored at -70°C overnight, then processed in the cold. Lanes 4, 5 and 6 were loaded with 10 μ g, 30 μ g and 100 μ g of protein, respectively.

Figure 2. Western blots using 6 different monoclonal antibodies. The gel was loaded with 5 μ g protein each of cytosol (lane 1) and nuclei (lane 2), of rat hippocampus.

Figure 3.

Photomicrographs of immunohistochemistry using antibodies that recognize different epitopes of the MR. MRN-12A7: hippocampus of intact and adrenalectomized rats probed with antibody 12A7 done in the same run. MRN-4E4: hippocampus from an intact rat probed with antibody MRN-4E4. 365-4D6: cerebellum probed with antibody rMR-365-4D6 using an Alexa 555 as the second antibody with nuclei counterstained with DAPI. Immunoreactivity is primarily nuclear and no axons are stained. MRN-3C9-A: cerebellum of an intact rat; immunofluorescence using antibody MRN-3C9; cytosol and axons are immunoreactive. MRN 3C9-B: rat heart at two magnifications; a, c, d- immunofluorescence, b- brightfield; d- coronary artery. MRN-4G5: cerebral cortex of an intact rat with marked labeling of axons and membranes.

Figure 4. Western blot of cytosol and nuclei of M1-rMR cells cultured overnight in serum free media and then incubated with media, aldosterone 3 nM, spironolactone 1 μ M and aldosterone 3 nM with spironolactone 1 μ M for 3 hr.